SEMI-ANNUAL PROGRESS REPORT

Prepared by K. S. Pilcher June 25, 1953

for period January 1 to June 30, 1953.

CONTRACT: Nonr-771 (00)

PROJECT: NR 135 186

ANNUAL RATE: $7805

CONTRACTOR: Oregon State College

PRINCIPAL INVESTIGATOR: K. S. Pilcher

Assistants: K. F. Soike
Victor Smith
Frances Trosper

TITLE OF PROJECT: A study of the effect of certain arginine analogs and other metabolite analogs on the multiplication of typical animal viruses.

OBJECTIVES: The objectives of this project may be stated as follows:

1. To examine the effect of certain structural analogs of arginine upon the multiplication of typical animal viruses, such as mumps, influenza, and equine encephalomyelitis, in the chick embryo. In view of the fact that arginine is an important constituent of virus proteins, as well as other proteins, it might influence the formation of virus protein. Furthermore it has been reported (1) that arginine, ornithine and lysine all inhibit the development of mumps and influenza virus in tissue culture.

2. To examine such arginine analogs as are already available as outlined in paragraph (1); and to synthesize new compounds of this type for study, within the limits of available personnel and time.

3. To determine the effect upon virus development of such other compounds which are analogs of known metabolites as may become available to us and which appear worthy of investigation.

TABLE A

[illegible] of Further Preliminary Tests with Various Organic Compounds for Possible Inhibitory Activity against Influenza Virus in the Chick Embryo

Compound Tested	Dose per Egg	Dose of Virus per Egg	Fraction of Eggs Showing Virus Hemagglutinin	Hemagglutinin Titer of Pooled Fluids from All Eggs in Group *
[illegible] biguanide hydrochloride	6 mg.	50 ID_{50}	8/8	120
[illegible]yl biguanide hydrochloride	12 mg.	"	0/3	0//
[illegible]yl guanylurea	2 mg.	"	6/7	60
[illegible]l guanylurea	25 mg.	"	1/6	0
[illegible] guanylurea	0.125 mg.	"	5/5	120
[illegible] phosphate	25 mg.	"	7/8	60
[illegible]ium versene	3 mg.	"	7/11	60
[illegible]aminopurine	5 mg.	"	8/8	160
[illegible] acid	50 mg.	"	7/8	240
[illegible]chlorophenoxyacetic acid	25 mg.	"	5/6	120
[illegible]hoxyacetic acid	10 mg.	"	8/8	480
None: Controls receiving 1 ml. carboxymethyl cellulose.	—	"	7/7	160
None: Controls receiving virus only	—	"	7/8	160

* Reciprocals of highest fluid dilutions giving complete hemagglutination
// Fluid dilutions below 1:20 were not tested, and fluids giving a negative reaction at this dilution are arbitrarily assigned a value of 0.
Eggs were incubated at 35°C. approximately 44 hours before virus titrations were made.

PROGRESS:

A. Examination of Compounds for Influence on Virus Multiplication:

1. Materials and Methods:

The methods employed during the period of this report have been essentially the same as those described in previous reports.

2. Results:

Evaluation of Compounds for Influence on the Development of Lee Influenza Virus in the Chick Embryo.

(1.) Results of preliminary tests of compounds for inhibitory activity.

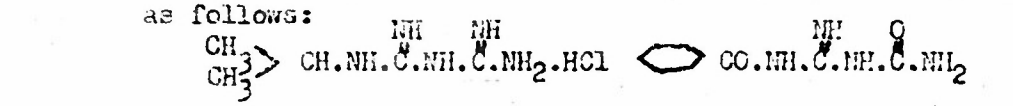
In the previous report covering the period July 1 to December 31, 1952, most of the work described the inhibitory effect of canavanine on the growth of the Lee influenza virus in the chick embryo and experiments designed to investigate the nature of this inhibition. One of the primary problems involved in this study was the source of supply of canavanine itself as this amino acid must be extracted from Jack bean meal by a laborious process. In view of the fact that the rate of progress of this work was limited by the supply it was decided to devote this period to a study of other compounds of potential interest while a stock pile of canavanine was being built up.

An interesting group of compounds bearing a structural relationship to arginine became available to us during this period. Samples were originally obtained through the kindness of Dr. I. F. Halverstadt of the Chemical Research Department of Cutter Laboratories. These compounds included guanylurea derivaties and biguanides. They were synthesized originally by the Product Development Department of Merk and Company, and further quantities have been obtained from the latter group. Additional compounds were also obtained through the courtesy of Dr. B. E. Christensen, Professor of Organic Chemistry, Oregon State College.

The results of the preliminary tests on the compounds examined during this period are presented in Table A. These tests were only intended to indicate whether a compound might have an influence on the development of the virus and whether it was worthy of further investigation. It will be noted that two of the compounds in this table appear to have had a rather striking inhibitory effect on the growth of the virus as judged by the hemagglutinin titer of the pooled allantoic fluids from the treated eggs. These are isopropyl biguanide hydrochloride and benzoyl guanylurea. As judged by the results of this test with a very small number of eggs, both of these compounds prevented

the development of sufficient virus to be detectable in the pooled fluids. These compounds obviously were worthy of further investigation.

The structure of these two compounds may be indicated as follows:



isopropyl biguanide hydrochloride — benzoyl guanylurea

Although they differ somewhat, it is obvious that they have in common the guanidino grouping which is of course found in arginine.

It will be noted also that three other compounds, i.e. cyclohexyl guanylurea, guanylurea phosphate and disodium versene also reduced the virus titers of the pooled allantoic fluids below the level of the controls. Statistically, significance cannot be attached to any of these differences, but the data suggest some slight activity by these compounds. The only other compound which produced a result which might be considered different from the control is naphthoxyacetic acid. The hemagglutinin level here was three times that of the control eggs, suggesting stimulation of virus development. However, we have attempted to confirm this result and have been unsuccessful. Apparently some conditions prevailed in this particular test which we have not been able to duplicate.

From the foregoing results it was concluded that an investigation of the effects of isopropyl biguanide hydrochloride and benzoyl guanylurea on development of the virus was justified.

(2) Further investigation of the inhibition of the Lee influenza virus by isopropyl biguanide* and benzoyl guanylurea.

The nature of the inhibition of the Lee virus by the above two compounds has been investigated fairly extensively. Most of the work however has been carried out with isopropyl biguanide because of the rather limited supply of benzoyl guanylurea, which could not be obtained in additional quantity.

To begin with, the meager data from the preliminary test was supplemented by further studies in which the fluids from individual eggs were titrated for virus hemagglutinin. The results of a typical experiment employing isopropyl biguanide are presented in Table I. In this experiment as in all others in this report unless indicated otherwise, injections of both virus and compound were made into the

*Hereafter the term isopropyl biguanide is used in this report it is understood that the compound is in the form of the

TABLE 1

Inhibitory Effect of Isopropyl Biguanide on the Influenza Virus in the Chick Embryo as Measured by the Hemagglutination Reaction

Egg No.	Hemagglutinin Titers of Fluids from Eggs Receiving 50 ID_{50} of Virus Only	Hemagglutinin Titers of Fluids from Eggs Receiving 10 mg. Isopropyl Biguanide an Hour before 50 ID_{50} of Virus
1	320*	20*
2	320	0#
3	320	0
4	240	0
5	240	0
6	240	0
7	160	0
8	80	0
9	40	0
10	0	0
Pool of Fluids from all Eggs in Group	160	0

* Reciprocals of highest fluid dilutions giving complete hemagglutination.

\# Fluid dilutions below 1:20 were not tested, and fluids giving a negative reaction at this dilution are arbitrarily assigned a value of 0.

Eggs were incubated at 35°C approximately 44 hours before virus titrations were made.

allantoic sac. It is evident from these results that the influenza virus is markedly inhibited by 10.0 mg. of isopropyl biguanide in the chick embryo for at least 44 hours after inoculation, as judged by the hemagglutinin titers.

The results of a similar experiment in which the dose of isopropyl biguanide was injected in the yolk sac of the chick embryo while the virus was injected into the allantoic sac are given in Table 2. From these data it seems apparent that it is not necessary to have direct contact between the compound and the virus in the allantoic sac in order to demonstrate inhibition of the virus. These results suggest that the compound may be absorbed into the circulation of the embryo and in some manner interfer with the growth of the virus in infected cells.

The results of more detailed observation of the effect of benzoyl guanylurea on the Lee influenza virus are shown in Table 3. From inspection of this table it also seems quite obvious that this compound too has a very marked inhibitory effect upon the development of the virus as judged by the hemagglutinin titer. In comparing results obtained with the two compounds it should be noted that a dose of 10.0 mg. of isopropyl biguanide disolved in a volume of 0.2 ml. of distilled water was regularly employed. This had been found to be about the maximum tolerated dose. However, benzoyl guanylurea has a much lower solubility and in the case of this compound it was necessary to employ a suspension made up to a concentration of 50 mg. per ml. in a suspending medium of one percent low viscosity carboxy-methyl cellulose. The maximum tolerated dose of the compound had been found to be about 25.0 mg. Thus it is hard to compare the activity of the two compounds weight for weight because of the large difference in solubility.

Experiments were next carried out to determine the minimum effective dose of isopropyl biguanide which could cause inhibition of the virus. Results of these experiments appear in Table 4. It will be noted that the inhibitory effect drops off rather rapidly with decreasing dosage. 10.0 mg. per egg inhibited hemagglutinin development completely. The inhibition produced by a dose of 5.0 mg. per egg was still marked and in the case of 2.5 mg. per egg was still significant. A slight effect may have resulted from the use of 1.0 mg. per egg, but the difference between the result obtained at this dose level and the control group is not significant.

Similar experiments were carried out with benzoyl guanylurea and these are given in Table 5. The effect of the decreased dosage is even more apparent in the case of this compound. 25.0 mg. per egg caused almost complete suppression of hemagglutinin whereas 12.5 mg. per egg did not cause any significant reduction in the hemagglutinin titer.

An effort was next made to determine over how long a [illegible] inhibitory effect of isopropyl biguanide could

TABLE 2

Inhibitory Effect of Isopropyl Biguanide Injected in the Yolk Sac on Lee Influenza Virus Injected in the Allantoic Sac of Chick Embryos.

Egg No.	Hemagglutinin Titers of Fluids from Eggs Receiving 10 $^{-5}$ Egg LD_{50} of Virus only in Allantoic Sac	Hemagglutinin Titers of Fluids from Eggs Receiving 10 mg. Isopropyl Biguanide in the Yolk Sac an Hour Before 50 EID_{50} of Virus in Allantoic Sac.
1	[illegible]	0
2	[illegible]	0
3	[illegible]	0
4	320	0
5	[illegible]	0
6	[illegible]	0
7	320	0
8	320	-
9	160	-
10	320	-
Pool of Fluids from all eggs in Group	480	0

* Reciprocals of highest fluid dilutions giving complete hemagglutinations

\# Fluid dilutions below 1:20 were not tested, and fluids giving a negative reaction at this dilution are arbitrarily assigned a value of 0.

Eggs were incubated at 35°C approximately 44 hours before virus titrations were made.

TABLE 3

Inhibitory Effect of Benzoyl Guanylurea on Lee Influenza Virus in the Chick Embryo as Measured by the Hemagglutination Reaction

Egg No.	Hemagglutinin Titers of Fluids from Eggs receiving 50 ID_{50} of Virus Only	Hemagglutinin Titers of Fluids from Eggs Receiving 25 mg. Benzoyl Guanylurea an Hour Before 50 ID_{50} of Virus.
1	640	40*
2	240	20
3	240	0#
4	240	0
5	240	0
6	160	0
7	160	0
8	120	0
9	120	-
10	80	-
Pool of Fluids from all Eggs in Group	320	0

* Reciprocals of highest fluid dilutions giving complete hemagglutination.
# Fluid dilutions below 1:20 were not tested, and fluids giving a negative reaction at this dilution are arbitrarily assigned a value of 0.
Eggs were incubated at 35°C approximately 44 hours before virus titrations were made.

TABLE 1

Effect of Dosage of Isopropyl Biguanide on the Inhibition of Ice Influenza Virus in the Chick Embryo

Dose of Isopropyl Biguanide per Egg	Fraction of Eggs Showing Virus Hemagglutinin	Number of Eggs Showing Titers of (0)	(20-80)	(120-320)	(640 & above)	Hemagglutinin Titers of Pooled Fluids from all Eggs in Group *
10.0 mg.	1/10	9	1	0	0#	0
5.0 mg.	4/10	6	3	1	0	30
2.5 mg.	3/7	4	1	1	1	120
1.0 mg.	9/10	1	3	5	1	240
0.5 mg.	10/10	0	2	6	2	320
Virus	9/10	1	2	5	2	320

All eggs received 50 ID_{50} of virus approximately an hour after the injection of the compound. They were then incubated at 35°C approximately 48 hours before virus titrations were made.

* Reciprocals of highest fluid dilutions giving complete hemagglutination.
# Fluid dilutions below 1:20 were not tested, and fluids giving a negative reaction at this dilution are arbitrarily assigned a value of 0.

TABLE 5

[illegible] on the Inhibition of Lee Influenza Virus [illegible]

[illegible]	Fraction of Eggs Showing Virus Hemagglutinin	Number of Eggs Showing Titers of (0)	(20-60)	(120-240)	(480 & above)	Hemagglutinin Titers of Pooled Fluids from all Eggs in Group *
[illegible] mg.	2/8	6	2	0	0#	0
12.5 mg.	10/10	0	0	9	1	120
6.3 mg.	9/10	1	2	6	1	80
None	10/10	0	1	8	1	160

All eggs received 50 LD_{50} of virus approximately an hour after the injection of the compound. They were then incubated at 35°C approximately 48 hours before virus titrations were made.

* Reciprocals of highest fluid dilutions giving complete hemagglutination.
\# Fluid dilutions below 1:20 were not tested, and fluids giving a negative reaction at this dilution are arbitrarily assigned a value of 0.

be detected. All of the previous observations had been made after a period of approximately forty-four hours of incubation at 35°C. Accordingly observations were made on both treated and control eggs on both the third and fourth day of incubation. These results appear in Table 6. It will be noted that after sixty-eight hours of incubation treated eggs still showed a level of virus hemagglutinin which was only about twenty per cent of that found in the untreated controls. After ninety-two hours of incubation the titer of the pooled fluids from the treated embryos was about half as great as the comparable control group. Although this difference has not been tested for statistical significance it seems doubtful that with the relatively small number of eggs involved, such a difference could be demonstrated. However, it is quite possible that further expansion of the number of observations would reveal such a difference. In any event it seems apparent that the inhibitory effect of a single dose of 10 mg. of isopropyl biguanide, which is very marked after forty-four hours of incubation, is still marked after sixty-eight hours and is at best very slight after ninety-two hours of incubation.

TABLE 6

Duration of Inhibiting Effect of Isopropyl Biguanide on Lee Influenza Virus in the Chick Embryo

	Fraction of Eggs Showing Virus Hemagglutinin	Number of Eggs Showing Titers of 0 (20-80)	(120-320)	(480 & above)	Hemagglutinin Titers of Pooled Fluids froms all Eggs in Group *
Eggs incubated 68 hours at 35°C after receiving 10 ID_{50} of Lee Virus	9/10	1	0# 5	4	320
Eggs incubated 68 hours at 35°C after receiving 10 mg. IBG+ and 10 ID_{50} of Lee Virus an hour later	9/9	0	8 1	0	60
Eggs incubated 92 hours at 35°C after receiving 10 ID_{50} of Lee Virus	10/10	0	0 5	5	480
Eggs incubated 92 hours at 35°C after receiving 10 mg. IBG+ and 10 ID_{50} of Lee Virus an hour later.	8/8	0	1 7	0	240

* Reciprocals of Highest fluid dilutions giving complete hemagglutination.
# Fluid dilutions below 1:20 were not tested, and fluids giving a negative reaction at this dilution are arbitrarily assigned a value of 0.
+ IBG = isopropyl biguanide

The influence of the dose of virus upon the inhibitory effect of isopropyl biguanide has been investigated to some extent, although additional work is required. Results obtained thus far are shown in Table 7. It will be noted in this table that the dose of virus has a definite influence upon the final result. When the virus inoculum is small, complete suppression of the virus hemagglutinin may be observed. When the dose of virus is increased the inhibitory effect becomes less marked. However, with a dose of ten thousand ID_{50}, which is the highest virus level so far tested, a slight inhibitory effect may still be noted. Further data bearing on the point will be obtained. However, the results obtained are in agreement with expectation, from what is known of the behavior of various infectious agents and inhibitory compounds.

A study has also been made of the influence of the time interval between injection of the virus and injections of isopropyl biguanide on the inhibition of the virus in the chick embryo. The data bearing on this point are presented in Table 8. It will be noted that there was no apparent difference in the end result if the compound was injected an hour before the virus or two hours after the virus. In both cases the development of virus hemagglutinin was almost completely suppressed. When the compound was administered twenty-four hours after the virus, the inhibitory effect was still marked and the virus hemagglutinin in treated eggs was only about ten per cent of that found in controls. When the injection of the compound was delayed until thirty six hours after infection with the virus a slight inhibitory effect was still found. When this time interval, however, was increased to forty-four hours no significant effect was demonstrable. It is interesting to note that the inhibition is still so striking, even when injection of the compound is delayed for twenty-four hours after infection. This observation suggests that the mechanism of inhibition is not concerned primarily with interference with adsorption of the virus to the susceptible cells, but is more likely related to some step in the formation of the virus itself or of its release from the cells.

A certain amount of similar data has been obtained with benzoyl guanylurea. This is much more limited because of the limited quantity of this compound which was available. These results appear in Table 9. It will be noted that here also essentially identical results were obtained if the compound was given either an hour before or two hours after the virus. In both cases the development of virus hemagglutinin was markedly suppressed. When the compound was given as late as twenty-four hours after the virus, however, only a slight inhibitory effect was noted. Thus the results with this compound are much less striking when treatment is delayed for twenty-four hours than is the case with isopropyl biguanide.

TABLE 7

Influence of Virus Dose Level on the Inhibitory Effect of Isopropyl Biguanide on Lee Influenza Virus[*] in the Chick Embryo

Dose of Virus Injected	Dose of Isopropyl Biguanide	Fraction of Eggs Showing Virus Hemagglutinin	Number of Showing Titers of 0(20-80)	(120-320)	640 & above	Hemagglutinin Titers[#] of Pooled Fluids from all Eggs in Group[#]
7 ID_{50}	10 mg.	0/10	10	0	0	0
7 ID_{50}	none	6/10	2	2	5	1	160
70 ID_{50}	10 mg.	5/10	5	5	0	0	80
70 ID_{50}	none	10/10	0	0	8	2	320
700 ID_{50}	10 mg.	7/10	3	1	5	1	160
1600 ID_{50}	none	10/10	0	0	4	6	1[illegible]0
10,000 ID_{50}	10 mg.	9/10	1	1	8	0	160
10,000 ID_{50}	none	9/10	1	0	7	2	320

All eggs that were to receive isopropyl biguanide were injected about an hour before the injection of the indicated amount of virus. They were then incubated at 35°C. approximately 48 hours before virus titrations were made.

In Exp. No. 1, lyophilized virus was used; in Exp. No. 2, fresh virus.

* Reciprocals of highest fluid dilutions giving complete hemagglutination.

\# Fluid dilutions below 1:20 were not tested, and fluids giving a negative reaction at this dilution are arbitrarily assigned a value of 0.

TABLE 8

Influence of Time Interval Between Infection and Injection of Isopropyl Biguanide on the Inhibition of Lee Influenza in the Chick Embryo.

10 mg. Isopropyl Biguanide Injected	Fraction of Eggs Showing Virus Hemagglutinin	Number of Eggs Showing Titers of 0	(20-80)	(120-320)	(480 & above)	Hemagglutinin Titers of Pooled Fluids from all Eggs in Group *
1 hour before virus	3/10	7	3	0	0#	0
2 hours after virus	1/10	9	1	0	0	0
24 hours after virus	9/18	9	7	2	0	30-40+
36 hours after virus	14/16	2	5	9	0	120+
44 hours after virus	10/10	0	2	4	4	320
Controls receiving virus only	20/20	0	1	9	10	320-480

All eggs received 50 ID_{50} of virus. 10 mg. Isopropyl biguanide was administered at the indicated times. Eggs were incubated at 35°C. for approximately 48 hours before virus titrations were made.

* Reciprocals of highest fluid dilutions giving complete hemagglutination.
\# Fluid dilutions below 1:20 were not tested, and fluids giving a negative reaction at this dilution are arbitrarily assigned a value of 0.
\+ Data represents combined results of 2 experiments.

TABLE 9

Influence of Time Interval Between Infection and Injection of Benzoyl Guanylurea on the Inhibition of Lee Influenza in the Chick Embryo.

25 mg. Benzoyl Guanylurea Injected	Fraction of Eggs Showing Virus Hemagglutinin	Number of Eggs Showing Titers of				Hemagglutinin Titers of Pooled Fluids from all Eggs in Group *
		0	(20-80)	(120-320)	(480 & above)	
1 hour before virus	4/10	6	4	0	0#	20
2 hours after virus	1/8	7	1	0	0	0
24 hours after virus	8/8	0	2	6	0	120
Controls receiving virus only	9/10	1	1	8	0	240

All eggs received 50 ID_{50} of virus. 25 mg. of benzoyl guanylurea was administered at the indicated times. This was suspended in a 1% aqueous solution of carboxymethylcellulose to give a concentration of 25 mg. per ml. Control eggs received 1 ml. of the suspending agent only an hour before the virus. Eggs were incubated at 35°C. for approximately 48 hours before virus titrations were made.

* Reciprocals of highest fluid dilutions giving complete hemagglutination.

\# Fluid dilutions below 1:20 were not tested, and fluids giving a negative reaction at this dilution are arbitrarily assigned a value of 0.

It is of course of interest to know whether these compounds have any direct inactivating effect on the Lee influenza virus in vitro. Experiments bearing on this point are recorded in Table 10. Isopropyl biguanide has been the only compound investigated and a concentration of 2 mg. per ml. has been employed, as this is estimated to be the maximum concentration produced in the allantoic fluid of the chick embryo when a dose of 10 mg. is administered. Periods of contact between the virus and the compound of two hours and twenty hours have been tested at temperatures of 10°C and 35°C. It will be noted from the table that in no case was there any effect upon the hemagglutinin titer of the virus. In the case of the virus infectivity, however, it is apparent that in the presence of the compound after twenty hours at 35°C this property of the virus deteriorated more rapidly than in a control preparation containing only the virus. The pH of the two preparations was approximately the same so that the difference noted could not be ascribed to a pH effect. At 10°C, after a twenty hour period of exposure a preparation containing isopropyl biguanide was slightly less active than the control but the difference can probably not be considered significant. Similarly after a period of two hours at 35°C the preparation containing isopropyl biguanide showed a slightly lower infectivity than the control preparation. Further experiments are planned to verify and extend these results. At present, however, it appears that isopropyl biguanide at 2 mg. per ml. may cause a reduction in infectivity of the virus over a twenty hour period of contact at 35°C. Since this effect is at best very slight within a two hour period at 35°C or within a twenty hour period at 10°C and since there appears to be no effect at all upon the hemagglutinating activity of the virus, it seems unlikely that a direct inactivating effect on the virus can account entirely for the inhibitory activity of the compound.

The effect of isopropyl biguanide upon the development of the Lee influenza virus has been measured by means of infectivity titrations as well as by hemagglutinin titrations. This method has not been used routinely because it is less precise and more laborious, but the inhibitory effect can be demonstrated by either method. Infectivity titers of virus from eggs treated with isopropyl biguanide have usually been at least one log unit lower than the titers found in untreated eggs.

Experiments have been carried out to examine the possible effect of isopropyl biguanide on the adsorption of the virus by chorio-allantoic membrane tissue in vitro. These experiments so far have failed to show that the compound in a concentration of 2 mg. per ml. interferes to any measurable extent with the adsorption of the virus by the membrane tissue.

TABLE 10

In Vitro Effect of Isopropyl Biguanide on Lee Influenza Virus

Exp. No.	Time and Temperature of Incubation		Hemagglutinin* Titer	No. of EID_{50}+ of Virus per ml.
1	None	Virus only before incubation	160	$10^{7.15}$
"	2 hours at 10°C.	Virus only	160	$10^{7.60}$
"	2 hours at 10°C.	Virus + IBG#; 2 mg. per ml.	160	$10^{7.40}$
"	2 hours at 35°	Virus only	160	$10^{7.74}$
"	2 hours at 35°C.	Virus + IBG; 2 mg. per ml.	160	$10^{7.17}$
2	None	Virus only before incubation	240	$10^{6.61}$
"	20 hours at 10°C.	Virus only	240	$10^{7.50}$
"	20 hours at 10°C.	Virus + IBG; 2 mg. per ml.	240	$10^{7.00}$
"	20 hours at 35°C.	Virus only	320	$10^{3.64}$
"	20 hours at 35°C.	Virus + IBG; 2 mg. per ml.	320	$10^{2.00}$

* Reciprocals of highest dilutions of fluid giving complete hemagglutination.

\# IBG = isopropyl biguanide

\+ EID_{50} = quantity of virus needed to infect 50% of inoculated chick embryos.

B. Preparation of Compounds for Antiviral Testing.

The work on an improved method for isolation of canavanine has been continued. Using slight refinements in the procedure some 35 g. have been prepared. Several more small scale attempts to isolate canavanine by the use of ion exchange resins have been made. Paper chromatography indicates that arginine and histidine follow canavanine through the columns. Colorimetric analysis indicates that canavanine is present in the acid eluate but attempts at isolation have been unsuccessful. It is thought that decomposition may occur on the ion exchange resin as has been reported to occur on an adsorption column (Archibald[1]).

To check this possibility pure canavanine was run through the resins. According to the colorimetric method the IRC - 50 resin buffered at pH4.7 retained 92% of the canavanine; when buffered at pH7.0 it retained 27%; and the IRA-400 resin retained 69%. However, the flavianate prepared from these acid eluates did not correspond to canavanine flavianate; this may be due to the formation of a eutectic mixture with ammonium flavianate or to flavianates of decomposition products of canavanine. This is being investigated further.

5-aminopyrimidinedione - 2,4; 5-nitropyrimidinedione - 2,4; 5-α,α,-dichloroacetamidopyrimidinedione - 2,4; 2-thio-4-oxypyrimidine, 2-methylmercapto-4-hydroxypyrimidine, cyanuric acid and biuret have been prepared by procedures described in the literature. The colorimetric determination for canavanine (Archibald[1]) has been extended to alcoholic solutions. Canaline has been made enzymatically and desaminocanavanine prepared by the method of Kitogawa[2].

Plans for the immediate future include the isolation of more canavanine and the preparation of certain of its derivatives.

SUMMARY:

1. Of a number of additional compounds examined two have been found to have a marked inhibitory effect on the development of the lee influenza virus in the chick embryo. These are isopropyl biguanide hydrochloride and benzoyl guanylurea. Both of these compounds contain the guanidino group and in this respect bear some structural relationship to arginine.

2. The inhibitory effect shown by isopropyl biguanide is equally marked when the compound is administered in the yolk sac or the allantoic sac. As the virus is injected into the allantoic sac in both cases this suggests that no direct contact between the virus and the compound in the allantoic sac is necessary in order for inhibition to be demonstrated.

1. Archibald, R. M.; J. Biol. Chem. 165, 169 (1946).
2. Kitogawa, M.; J. Biochem. (Japan), 25, 23 (1941).

3. In the case of isopropyl biguanide 10.0 mg. per egg, which is about the maximum tolerated dose, produced the most marked inhibition of the virus. However, a significant inhibition is noted with a dose as low as 2.5 mg. per egg. In the case of benzoyl guanylurea 25.0 mg. per egg produced the maximum degree of inhibition, whereas 12.5 mg. per egg was ineffective. This compound was employed in the form of a suspension because of its low water solubility; hence it is not possible to make direct comparisons between the two compounds for activity on a weight basis.

4. In the case of isopropyl biguanide the inhibitory effect of a single dose of 10 mg. is still evident after the treated eggs have been incubated for three days at 35°C. Even after four days of incubation a slight effect is still apparent.

5. The inhibitory effect of isopropyl biguanide is definitely influenced by the dose of virus administered. With doses of about ten to one hundred ID_{50} the development of virus hemagglutinin is very markedly suppressed. With a dose of ten thousand ID_{50} slight inhibition is noted.

6. Isopropyl biguanide may be administered as long as twenty four hours after the infecting virus and still produce a marked suppression of virus hemagglutinin. Even after thirty-six hours a noticeable inhibition is found. This observation seems to indicate that the inhibitory effect is not due to interference with adsorption of the virus by the cells.

7. In the case of benzoyl guanylurea the inhibition of the virus is equally marked if the compound is given an hour before the virus or two hours after the virus. If injection of the compound is delayed for twenty-four hours, however, the effect is slight.

8. Isopropyl biguanide at a concentration of 2 mg. per ml. has no effect upon the hemagglutinating activity of the Lee influenza virus after exposures as long as twenty hours at 35°C. It does, however, apparently cause a more rapid loss of infectivity than is noted in a control preparation containing only the virus. This effect upon infectivity is at best very slight in twenty hours at 10°C or in two hours at 35°C. It seems unlikely that it can entirely account for the inhibitory activity of the compound in the development of the virus.

9. Isopropyl biguanide has been shown to inhibit the development of Lee influenza virus in the chick embryo using infectivity as a measure of virus concentration, as well as the hemagglutinin measurement. Eggs treated with the compound have shown a virus titer at least one log unit lower than untreated eggs.

10. Experiments carried out in vitro have failed to show that isopropyl biguanide has any influence upon the adsorption of the Lee influenza virus by the chorio-allantoic membrane.

11. A considerable amount of effort has been devoted to improving the extraction and purification of canavanine from jack bean meal. Ion exchange resins have been tried but a successful method has not been achieved. Altogether some 35 grams of canavanine have been prepared by the original laborious process.

12. Additional compounds which have been prepared by synthesis or isolation include, desaminocanavanine, canaline, cyanuric acid, 5-aminopyrimidinedione - 2,4; 5-nitropyrimidinedione - 2,4; 2-thio-4- oxypyrimidine; 5-α,α,-dichloroacetamidopyrimidinedione - 2,4; and 2-methylmercapto-4-hydroxypyrimidine.

Plans for further work and publications.

The study of the effect of isopropyl biguanide on the development of the influenza virus is not yet complete. Some of the phases of the work reported above require additional data. Also attempts are planned to reverse the inhibition by means of various possible metabolites which might be involved. It is hoped that this may shed some light on the mechanism of the inhibition. This study is to be carried out in tissue culture as well as in the chick embryo. Work has also been started to examine the effect of this compound upon influenza virus infections in mice.

The compound will also be tested for possible effects upon influenza A virus, mumps virus, equine encephalomyelitis virus and possibly others.

It is also planned to complete the study of canavanine which has also been found to be inhibitory to the Lee influenza virus as was described in the previous report covering the period from July 1 to December 13, 1952.

The only publication of this work submitted so far has been the abstract which appeared in the first volume of Progress Report Abstracts; Microbiology Branch; Office of Naval Research. It is planned, however, to present the work described in this report at the national S.A.B. meetings in San Francisco in August 1953, and to submit a written report of this work for publication soon thereafter.